USE OF GELATIN TO REMOVE INTERFERENCE BY SERUM WITH THE SOLID PHASE ENZYME-LINKED SANDWICH IMMUNOASSAY OF INSULIN

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1. Introduction

An enzyme-linked sandwich immunoassay system using the antibody Fab′–β-D-galactosidase complex and antibody-bound glass or silicone rubber pieces as solid phases is highly sensitive [1–4]. Even attomole amounts of macromolecular antigens are measurable in specified conditions [3]. However, the assay system is interfered with non-specifically by serum factors, when serum is present in the sample being analysed [4]. Interference by serum factors has been reported with both radioimmunoassay [5,6] and enzyme immunoassay [7], especially when solid phases are used as a separation technique.

Here, we describe a method to remove interference by serum with the enzyme-linked sandwich immunoassay of insulin. Inclusion of gelatin, a hydrophobic protein, with a relatively high concentration of salt in the assay mixture is found to remove effectively interference by serum with the sandwich immunoassay of insulin.

2. Materials and methods

Crystalline beef insulin was from Fluka AG (23-25 U/mg), and porcine insulin from Novo Industri A/S (Actrapid, 40 U/ml). Guinea pig (anti-beef insulin) serum was from Miles Labs. The immunoglobulin G (IgG) fractions from the anti-serum were prepared by fractionation with Na_2SO_4 [8] followed by passage through a column of DEAE-cellulose (DE 52, Whatman Biochemicals Ltd) [9]. $F(ab')_2$ fragments of the IgG fractions of the anti-insulin serum were prepared by digesting the IgG with pepsin (Sigma Chemical Co.) as in [10].

2.1. *Guinea pig (anti-beef insulin) Fab′–β-D-galactosidase complex*

The $F(ab')_2$ fragments from the anti-serum were reduced with 2-mercaptoethylamine and coupled to β-D-galactosidase from *E. coli* (Boehringer Mannheim) using *N,N′-o*-phenylenedimaleimide [11]. The amounts of the complex are expressed as units of enzyme activity, and 1 unit of activity is defined as that which hydrolyses 1 μmol substrate/min under the conditions described.

2.2. *Guinea pig (anti-insulin) IgG-bound solid phases*

The IgG fractions of anti-insulin serum were immobilized non-covalently on silicone rubber (string, 3 mm diam. from Sanko Plastic Co., Osaka, cut into 4 mm lengths) or polystyrene beads (3.2 mm diam., Precision Plastic Ball Co., Chicago) as in [12], and the pieces stored in 0.01 M sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl, 1 mM $MgCl_2$, 0.1% bovine serum albumin (fraction V, Armour Pharmaceutical Co.) and 0.1% NaN_3 (buffer A) at 4°C for at least 1 week prior to use.

2.3. *Sandwich procedures for insulin assay*

A piece of the antibody-bound silicone rubber or polystyrene bead was incubated with various amounts of insulin, or with 2.5–100 μl human serum in 0.5 ml final vol. buffer A. When gelatin was involved in the reaction mixture, 0.4 ml portions of the above medium

were replaced by buffer G (0.01 M sodium phosphate buffer (pH 7.0) containing 0.3 M NaCl, 1 mM $MgCl_2$, 0.1% bovine serum albumin, 0.5% gelatin from Difco Labs and 0.1% NaN_3). After incubation at 30°C for 2 h with shaking, each piece was washed twice at 20–25°C with 1 ml of the corresponding buffer (buffer A or G). The solid phase was then incubated at 4°C overnight with 3 munits of the (anti-insulin) Fab′–β-D-galactosidase complex in a volume of 0.2 ml of buffer A. Each piece was washed twice with buffer A and the enzyme activity bound was assayed in buffer A as in [1] using 4-methylumbelliferyl-β-D-galactoside (Sigma Chemical Co.) as substrate.

3. Results and discussion

Standard curves for the sandwich immunoassay of insulin both in buffer A and buffer G are shown in fig.1. A linear dose–response of the enzyme activity bound on the solid phase was observed between 0.25 and 8 μU insulin in both, although the increased salt concentration in the first immunoreaction in buffer G resulted in a slight decrease in enzyme activity bound. Assay with porcine insulin and beef insulin gave the same result.

The effect of serum samples on the immunoassay was examined by adding 50 μl normal human serum to each of the assay mixtures, without or with 1 μU insulin, and the recovery of the added insulin was calculated from the corresponding standard curves. As shown in table 1, poor recovery in various degrees was observed in the assay with buffer A alone. However, the recovery was satisfactory for each serum sample when assayed with buffer G. Various amounts of insulin (0.25–8.0 μU) added with 50 μl of serum were also completely recovered when assayed with buffer G (not shown).

The interference by serum with the assay in buffer A shown in table 1, in which silicone rubber pieces were used as solid phase, was also observed in the assay with antibody-bound polystyrene beads

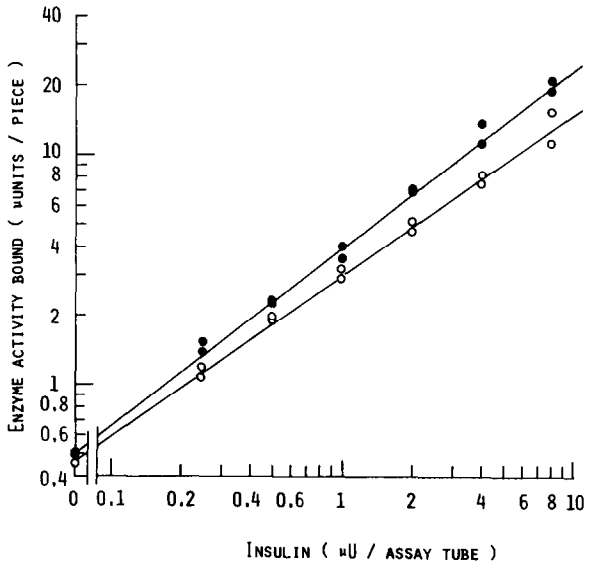

Fig.1. Standard curves for insulin estimation by sandwich enzyme-linked immunoassay. The (anti-insulin) antibody-bound silicone rubber was incubated with porcine insulin in buffer without (–●——●–) or with (–○——○–) gelatin, as described in the text.

Table 1
Recovery of insulin added to serum samples

Serum no.	Insulin recovered (μU)	
	a	b
2	1.14	0.36
3	0.85	0.19[a]
5	1.25	0.51
6	0.94	0.52
11	0.96	0.59
12	0.96	0.23[a]
13	1.07	0.10[a]
15	0.99	0.76
17	0.92	0.63
23	0.95	0.03[a]
means ± SD	1.00 ± 0.12	0.39 ± 0.25

[a] Without added insulin, enzyme activities bound to the solid phases were less than those bound in the absence of insulin in the standard curve

The enzyme-linked sandwich immunoassay of insulin was carried out with 10 different normal human serum samples in buffer without (b) or with (a) gelatin as described in the text. Each serum sample (50 μl) was subjected to the assay with and without addition of 1 μU porcine insulin, and the recovery of the added insulin was calculated from the corresponding standard curve. Values are means of duplicate assay

Table 2
Serum levels of insulin determined with different serum sample volumes

Serum no.		Level of insulin (μU/ml serum) determined with					
		2.5 μl	5 μl	10 μl	20 μl	50 μl	100 μl
4	a			46	44	46	46
	b			26	24	22	18
9	a			24	26	24	19
	b				11	6.4	7.2
29	a	340	320	330	320		
	b	260	220	240	160	120	

Each serum sample in various volumes was subjected to the assay in the buffer without (b) or with (a) gelatin as described in the text. Each value is the mean of duplicate assay

(0.38 ± 0.16 μU of recovery, 7 serum samples), and the interference was removed in the assay with buffer G (1.00 ± 0.11 μU of recovery).

To confirm the removal of interference by serum in the assay with gelatin, serum insulin levels were determined with serum samples of different volumes. As shown in table 2, a constant value of the insulin level was obtained in each serum in the assay with gelatin. On the other hand, the values obtained in the assay without gelatin were lower and tended to decrease with increasing serum sample volumes. These results indicate that the addition of μl volumes of serum in the assay mixture (0.5 ml) resulted in an appreciable interference with the assay in buffer A, and that such a sensitive inhibitory effect was completely removed by using the buffer containing gelatin in the assay.

To obtain complete removal of the interference, addition of gelatin with a high concentration of salt was essential, suggesting that a hydrophobic interaction between gelatin and the interference factors in the serum may aid removal of the inhibitory effect of these factors on the immunoreaction on the solid phase. An optimal concentration of NaCl in the assay mixture seems to be around 0.3 M, since a higher concentration, > 0.3 M in buffer G, brought about a stronger inhibition of the immunoreaction, lowering the sensitivity and the accuracy of the assay. Replacement of gelatin in buffer G by bovine serum albumin or egg albumin was ineffective.

Preliminary experiments indicate that the effect of gelatin is not peculiar to the insulin assay system but is also observed in several other sandwich assay systems tested in which the antibody–β-D-galactosidase complex and antibody-bound silicone rubber, glass, or polystyrene were used.

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